Synergistic effect of *Glomus intraradices* and *Frankia* spp. on the growth and stress recovery of *Alnus glutinosa* in an alkaline anthropogenic sediment

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Abstract

The presence of actinorhizas and arbuscular mycorrhizas may reduce plant stresses caused by adverse soil conditions. A greenhouse experiment was conducted using a sediment with a high pH, resulting from the disposal of waste originated at an acetylene and polyvinylchloride factory, in which Black alder (*Alnus glutinosa*) seedlings were inoculated either with *Glomus intraradices* BEG163 (originally isolated from the same sediment), *Frankia* spp. or both symbionts. After a 6-month growth period, plants inoculated with both symbionts had significantly greater leaf area, shoot height and total biomass when compared with the uninoculated control, the *Frankia* spp. and the *G. intraradices* treatments alone. In dual inoculated plants the N and P leaf content was significantly increased. A defoliation experiment was performed to evaluate the stress recovery of *A. glutinosa* and plants inoculated with both symbionts had a faster leaf regrowth and produced greater numbers of leaves. The dual inoculation resulted in greater numbers of and larger root nodules than when inoculated with *Frankia* spp. alone. The length and NADH diaphorase activity of the extraradical mycelium of *G. intraradices* was also significantly greater when dual inoculation was performed. The inoculation with *Frankia* spp. alone was shown to improve *A. glutinosa* growth, whereas *G. intraradices* alone had no positive effect under these environmental conditions. However, when the two symbionts were inoculated together a synergistic effect was observed resulting in a greater benefit for the plants and for both symbionts. The relevance of these findings for the phytorestoration of anthropogenic stressed sediments with high pH is discussed.

Introduction

Species of *Alnus* have been planted for many decades for erosion control, soil improvements, reclaiming strip mine areas and revegetating and sustaining roadsides (Cervantes and Rodríguez-Barrueco, 1992; Schwencke

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and Carú, 2001). *Alnus* spp. can grow and improve soil fertility due to their ability to fix atmospheric nitrogen in natural and disturbed soils (Hibbs and Cromack, 1990; Wheeler and Miller, 1990). Black alder (*Alnus glutinosa*) is known not only to form mycorrhizas with arbuscular mycorrhizal fungi (AMF), but also actinorhizas with nitrogen fixing *Frankia* (Struková et al., 1996). The mycorrhizal *Alnus* roots may be advantageous in infertile areas by enhancing nutrient uptake in those environments (Monzón and Azcón, 2001). Trees harbouring AMF and *Frankia* may have the advantage of high tolerance to environmental stresses (Ruskin, 1984; Sylvia and Williams, 1992). A dual symbiosis located in the roots, between plants and microorganisms capable of fixing dinitrogen and improving phosphate absorption from the soil may represent a great advantage to *Alnus* spp. Such plants may develop well in unfavourable environments (Berliner and Torrey, 1989). There have been reports of interactions between AMF and *Frankia* resulting in better growth of the host plant compared with the effect of a single symbiont (Chatarpaul et al., 1989; Fraga-Beddiar and Le Tacon, 1990; Russo et al., 1993; Tian et al., 2002). Jha et al. (1993) confirmed that isolates of *Glomus mosseae* and *Frankia* in combination with *Alnus nepalensis* caused an increase in nodule dry weight and nitrogenase activity over a range of phosphate treatments. *Frankia*, on the other hand, increased colonisation by AMF. One of the important advances in the last decade of mycorrhizal research has been studies of the structure, organisation and function of the extraradical mycelium (ERM) (Vosátka and Dodd, 1998; Boddington et al., 1999; Dodd et al., 2000).

However, no attention has been given to the interactions between *Frankia* and the ERM development. The beneficial effects may vary according to the combination of plant species, *Frankia*, mycorrhizal fungi strains and substrate (Isopi et al., 1994). The efficacy of different endosymbiont-plant genotype combinations may not be predictable in different soils and habitats; thus, to check the efficiency of a symbiotic association, applied studies are required. In such studies, the effect of inoculation with AMF and *Frankia* will depend on the environmental conditions, including soil pH, salinity and nutrient levels (Cervantes and Rodríguez-Barrueco, 1992). The presence of AMF in highly alkaline sediments has been reported (Oliveira et al., 2001) but their role in promoting plant growth, nutrient uptake and stress recovery under such extreme conditions remains unclear. Many authors have reported relatively narrow ranges of pH for presence or activity of specific AMF in soils (Robson and Abbott, 1989), and information about AMF function over broad soil and growth media pH ranges is limited (Clark and Zeto, 1996). High pH soils are usually calcareous and arid, have poorly weathered clays, high concentrations of soluble salts and sustain sparse or specialized kinds of vegetation (Clark and Zeto, 1996). Highly alkaline conditions in soils and sediments can be created anthropogenically due to different industrial activities, generating large areas that need to be restored (Ash et al., 1994; Kernaghan et al., 2002). *A. glutinosa* was the plant species with greater survival rate in a field trial with *A. glutinosa*, *Salix atrocinerea* and *Acer negundo* planted in a highly alkaline anthropogenic sediment (Oliveira et al., unpublished). Since highly alkaline sediments are very difficult environments for plants, our approach was to use *A. glutinosa* together with inoculated AMF and actinomycete symbionts as a model for the phytorestoration of anthropogenic sediments. The purpose of this work was to study the role of the interactions between *Frankia* spp. and *Glomus intraradices* BEG163 (isolated from the sediment) on *A. glutinosa* growth and stress recovery in a highly alkaline anthropogenic sediment.

Materials and methods

Site and sediment characteristics

The study site was a 10 ha anthropogenic sedimentation pond located in the industrial complex of Estarreja, Northern Portugal (40°46′30″ N, 08°35′04″ W) that has been created by the deposition of 300 000 ton of solid waste residues from the production of acetylene and polyvinylchloride during a 26-year period. The site had scant vegetation, comprising mainly dwarfed *Pinus pinaster* Ait., *Salix atrocinerea* Brot. and herbaceous plant species such as *Conyza bilbaoana* J. Rémy and *Pseudognaphalium luteo-album* (L.) Hilliard and B.L. Burtt. Field collected sediment from the uppermost 5 cm layer was sieved through a 3 mm mesh and autoclaved twice (121 °C for 25 min) on two consecutive days. Sub-samples were air-dried and sieved through a 2 mm mesh for the assessment of its chemical and physical properties. Extractable phosphorus was determined spectrophotometrically (Unicam, UV4-200, Cambridge, UK) (Olsen et al., 1954). Extractable potassium, sodium and calcium ions were determined by flame AES (Unicam, AA 9200X, Cambridge, UK). Extractable magnesium was determined by flame AAS (Unicam, AA 9200X, Cambridge, UK). Total Ca was determined by flame AES (Moore and Chapman, 1986). For carbon and nitrogen, sub-samples were sieved through a 0.1 mm mesh and were determined by element analysis (Elemental Analyser Carlo Erba, NC2500, Italy) (Monar, 1972).

Experimental design

The greenhouse experiment was conducted following a completely randomised experimental design divided

into four treatments: non-inoculated controls, plants inoculated with *G. intraradices* BEG163, plants inoculated with *Frankia* spp. and plants inoculated with both microbial symbionts. All treatments were replicated 20 times. *A. glutinosa* (L.) Gaertn. seeds were surface sterilized with 0.5% (v/v) NaOCl for 15 min. They were pre-germinated in plastic trays placed in a controlled greenhouse (photoperiod of 16 h, with supplementary metal halide 400 W lighting, temperature and relative humidity varied between 15 and 39 °C and 60% and 85%, respectively) in a mixture of fine sand and attapulgite clay (Agsorb 8/16, Oil-Dry Ltd., Wisbech, UK) (1:1) autoclaved twice (121 °C for 25 min) on consecutive days. After 42 d, equally developed seedlings were transplanted singly into 200 ml pots containing the alkaline sediment. A nitrocellulose membrane filter (24 mm diameter and 0.40 μm pore size) (Pragopore, Pragochema Ltd., Czech Republic) was inserted vertically in each pot for future measurements of the ERM lengths (Baláz and Vosátka, 2001). Microbial populations from the original non-sterile sediment were reintroduced to all pots by adding 10 ml of filtrate (Whatman No 1) from 100 g of sediment shaken for 2 h in 1 l of autoclaved deionised water. The plants were grown in the same controlled greenhouse of the Institute of Botany in Pruhonice, Czech Republic from December 2001 to June 2002 and watered every 2 d with deionised water. The pots with different inoculation treatments were periodically rotated to different bench positions to minimize differences due to their location in the greenhouse.

Microbial symbionts and inoculations

The AM fungus used in this experiment was *G. intraradices* BEG163 that had been isolated from the studied site. It was grown for 8 months in expanded clay (Leca 0/2, Leca Portugal, Avelar, Portugal) with *Trifolium pratense* as a host plant in open multispore pot culture. The resulting inoculum contained about 240 AMF propagules per ml, estimated by the most probable number method (Porter, 1979). *Frankia* spp. nodules were collected from a healthy *A. glutinosa* tree in Pruhonice, Czech Republic and stored at 4 °C for one week. Immediately before inoculation, separate lobes of *Frankia* nodules were washed with water, surface sterilized with 5% (v/v) NaOCl for 5 min and homogenized in a blender. The suspension (66.7 g nodules per l of autoclaved deionised water) was filtered through filter paper (Whatman No 1). Each pot from the mycorrhizal treatments received 5 g of AMF inoculum consisting of colonised root fragments, hyphae and spores in expanded clay placed 2 cm below the root system. This corresponded to about 1200 AMF propagules per pot. Every pot from the non-mycorrhizal treatments received 5 g of AMF inoculum autoclaved twice (121 °C for 25 min) in consecutive days. In order to eliminate differences in microbial populations introduced with the AMF inoculum, 5 ml of a suspension of the AMF inoculum were added to all pots from the non-mycorrhizal treatments (Koide and Li, 1989). The suspension was prepared as described above for the sediment. Each pot from the *Frankia* spp. treatments received 10 ml of the *Frankia* filtrate described above, at the base of the seedling. All the pots without *Frankia* spp. inoculation received 10 ml of autoclaved *Frankia* spp. filtrate. All inoculations were made at transplanting.

Plant growth and analyses

After a growth period of 6 months the number of leaves, shoot heights and root collar diameters were evaluated. The leaves were manually separated from the stems and the total leaf area was measured with a light area meter (LI-3100, LI-COR, Lincoln, Nebraska, USA). For chlorophyll analysis, fresh circular discs were cut with a 10.5 mm corer from the centre of the second mature leaf collected from the apex and extracted in *N*,*N*′-dimethylformamide and chlorophyll $a + b$ content determined according to Wellburn (1994). After leaf removal, all the plants were kept in the greenhouse to evaluate leaf regrowth (see defoliation experiment below). When the defoliation experiment was concluded, roots were gently shaken to remove sediment. The cohesive layer of sediment still attached to the roots that could only be removed after a second more thorough shaking was considered to be the rhizosphere sediment. The pH of the rhizosphere was measured in ultra-pure water. Total dry weight of plants was determined after drying the plant material at 70 °C for 48 h. Oven-dried leaves were finely ground and 0.3 g of material were digested according to Novozamsky et al. (1983). Total P and N were determined by colorimetry (Unicam, Helios Gamma, Cambridge, UK), total K was determined by flame AES (Jenway, PFP7, Jencons, UK) and total Ca by flame AAS (Unicam, AA 969 Solaar, Cambridge, UK) (Walinga et al., 1989). C/N ratio was determined by element analysis (Elemental Analyser Carlo Erba, NC2500, Italy) (Monar, 1972).

Defoliation experiment

The alders without leaves were left in the greenhouse for 15 d, after which a second leaf harvest was conducted with the newly produced leaves and the following parameters were evaluated: number of leaves, total leaf area and total leaf dry weight. The aim of this defoliation experiment was to investigate in which treatment the alders, growing in an extremely alkaline sediment, were more resistant to an imposed environmental stress and to determine their ability to recover after defoliation.

Frankia and my corrhizal parameters

Frankia nodules were counted and excised from the fresh roots. A multi-lobed nodule with a common point of emergence from the parent root was counted as one nodule. The dry weight was determined by drying the nodules at 70 °C for 48 h. Fresh root samples were stained with 0.05% (w/v) trypan blue in lactoglycerol (Koske and Gemma, 1989) and root colonisation by AMF was assessed using the grid-line intersect method (Giovannetti and Mosse, 1980). The sediment from each pot was homogenised after plant growth and AMF spores were isolated from 10 g of air-dried samples of sediment using a technique for wet-sieving and decanting (Gerdemann and Nicholson, 1963). The supernatant was filtered through a filter paper (Whatman No 1) with a 1 cm^2 grid. The number of spores of AMF was assessed under a stereo-microscope (Olympus SZ60, Japan) and identity confirmed. The ERM lengths were evaluated by two different methods: inserted membrane technique (IMT) (Baláz and Vosátka, 2001) and a modified membrane filtration technique (MFT) (Jakobsen et al., 1992). For both methods, the total lengths of ERM were assessed using the grid-line intersect method under a compound microscope (Olympus BX60, Japan), using an ocular grid at 200× magnification (Brundrett et al., 1994). The background lengths of mycelium found in non-mycorrhizal treatments were subtracted from all values obtained in the corresponding mycorrhizal treatments. For IMT the result was expressed in cm of hyphae per 1 cm^2 of the inserted membrane filter and for MFT the result was expressed in cm of hyphae per 1 g of air-dried sediment. The NADH diaphorase activity (Sylvia, 1988; Hamel et al., 1990) was determined in the remaining ERM extracted from the sediment by wet-sieving. The proportion of ERM length that contained purple precipitate was estimated under a compound microscope at 200× magnification.

Statistic alanalysis

All the data were tested for normality and non-normally distributed data were square root transformed (Zar, 1999). The data from the mycorrhizal and *Frankia* parameters were analysed without including the respective control treatments using Student's *t*-test at a significance level of $P < 0.05$. All the remaining data were analysed using two-way analysis of variance (ANOVA) and when a significant F value was obtained ($P < 0.05$), treatment means were compared using the Duncan's multiple range test. Regression analysis of the ERM lengths evaluated by IMT and a modified MFT was performed at a significance level of 0.05. All statistical analyses were performed using the SPSS 11.0.0 software package.

Results

Sediment characteristics

The pH values were found to be very high (between 11.6 and 12.1), the sediment was considered to be saline based on the high conductivity values obtained and have low levels of extractable P (Table 1).

Plant growth and nutrition

Inoculation with *Frankia* spp. alone had a greater effect than *G. intraradices* alone on the growth parameters measured. When both symbionts were inoculated together, the total leaf areas, shoot heights, root collar diameters and total plant dry weights were significantly greater when compared with those inoculated with only one symbiont or the uninoculated controls (Table 2). Plants inoculated with *Frankia* spp. alone significantly increased the leaf N content by 197% compared with the uninoculated controls. This significant increase in leaf N content was even greater in dual inoculated plants since there was an increase of 277% compared with the uninoculated controls (Table 3). The leaf C/N ratio was significantly lower (i.e., higher relative N content) in plants inoculated with both symbionts when compared with all the other treatments. There was no significant effect of each of the symbionts inoculated individually on leaf P content when compared with the uninoculated controls. However, there was a significant increase of 240% on the leaf P content in plants

Table 1
Chemical and physical characteristics of the anthropogenic sediment

pH (H_2O)[a]	11.8
pH ($CaCl_2$)[a]	11.6
pH (KCl)[a]	12.1
Conductivity (μS cm^{-1})[b]	5980
Total C (%)[c]	8.91
Total organic C (%)	4.12
Total N (%)[c]	0.23
Total Ca (mg kg^{-1})[d]	12656
Extractable values (mg kg^{-1})	
P[e]	10.5
K[f]	52.0
Na[f]	19.0
Mg[f]	60.4
Ca[f]	5721

[a] 1:2.5 w/v.
[b] 1:5 w/v.
[c] Element analysis.
[d] Digestion with HNO_3 + HCl + H_2O_2.
[e] Olsen's $NaHCO_3$.
[f] Extraction with neutral NH_4CH_2COOH.

Table 2
Effect of *Frankia* spp. and *G. intraradices* on the growth of *A. glutinosa* in a highly alkaline anthropogenic sediment

Frankia	AMF	Number of leaves	Total leaf area (cm^2)	Shoot height (cm)	Root collar diameter (mm)	Total plant dry weight (g)
Non-inoculated	Non-inoculated	11 ± 1.1ab	99.8 ± 5.9a	20.8 ± 0.6a	5.0 ± 0.1b	1.69 ± 0.06a
	G. intraradices	9 ± 0.4a	68.2 ± 2.8a	19.1 ± 0.6a	3.9 ± 0.1a	1.39 ± 0.05a
Frankia spp.	Non-inoculated	13 ± 0.6bc	249.9 ± 11.5b	28.8 ± 0.9b	7.3 ± 0.1c	4.39 ± 0.16b
	G. intraradices	14 ± 1.0c	340.9 ± 20.4c	33.4 ± 1.2c	8.0 ± 0.2d	4.95 ± 0.18c
Frankia (A)		***	***	***	***	***
AMF (B)		NS	*	NS	NS	NS
A × B		NS	***	***	***	**

The values are means of 20 replicates ± SEM. Means followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $P < 0.05$. For two-way ANOVA: *significant effect at the level of $P < 0.05$, **significant effect at the level of $P < 0.01$, ***significant effect at the level of $P < 0.001$, NS—non-significant effect.

Table 3
Effect of *Frankia* spp. and *G. intraradices* on *A. glutinosa* leaf content of N, P, K, Ca, Chlorophyll ($a + b$), C/N ratio and rhizosphere pH after a 6-month growth period

Frankia	AMF	N ($mg\ g^{-1}$)	C/N ratio	P ($mg\ g^{-1}$)	K ($mg\ g^{-1}$)	Ca ($mg\ g^{-1}$)	Chlorophyll $a + b$ ($mg\ g^{-1}$)	Rhizosphere pH
Non-inoculated	Non-inoculated	11.1 ± 2.2a	40.56 ± 4.3a	1.0 ± 0.2a	8.4 ± 0.6b	28.4 ± 9.7a	3.41 ± 0.3a	8.16–8.44a
	G. intraradices	9.3 ± 0.8a	45.12 ± 1.7a	1.1 ± 0.1a	6.6 ± 0.3a	33.5 ± 3.7a	3.04 ± 0.4a	8.03–8.32a
Frankia spp.	Non-inoculated	21.9 ± 1.7b	25.50 ± 0.3b	0.8 ± 0.2a	8.3 ± 0.6b	22.1 ± 3.8a	16.29 ± 0.9b	8.01–8.29a
	G. intraradices	30.8 ± 2.8c	14.48 ± 0.6c	2.4 ± 0.4b	10.8 ± 0.4c	20.2 ± 3.0a	18.12 ± 0.6c	7.03–7.39b
Frankia (A)		***	***	*	**	NS	***	***
AMF (B)		NS	NS	**	NS	NS	NS	***
A × B		*	**	**	**	NS	NS	***
n		5	5	5	5	5	20	10

For N, P, K and Ca, the values are expressed in mg of mineral per g of oven-dried leaf and for chlorophyll ($a + b$) in mg per g of fresh leaf. The pH values are ranges. Means ± SEM and pH range values followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $P < 0.05$. For two-way ANOVA: *significant effect at the level of $P < 0.05$, **significant effect at the level of $P < 0.01$, ***significant effect at the level of $P < 0.001$, NS—non-significant effect.

inoculated with both symbionts (Table 3). There was a significant increase of 129% of the leaf K content in plants inoculated with both symbionts simultaneously when compared with the uninoculated controls. There was no significant effect of inoculation with *Frankia* spp. and *G. intraradices* either individually or simultaneously on *A. glutinosa* leaf Ca content. The chlorophyll $a + b$ content in *A. glutinosa* leaves ($mg\ g^{-1}$ fw) was

Table 4
Effect of *Frankia* spp. and *G. intraradices* on the production of new leaves in *A. glutinosa* after experimental defoliation

Frankia	AMF	Number of leaves	Total leaf area (cm^2)	Total leaf dry weight (g)
Non-inoculated	Non-inoculated	8 ± 1.4a	6.8 ± 1.2a	0.033 ± 0.007a
	G. intraradices	6 ± 1.2a	2.3 ± 0.7a	0.018 ± 0.003a
Frankia spp.	Non-inoculated	2 ± 0.6a	4.8 ± 1.1a	0.022 ± 0.007a
	G. intraradices	23 ± 4.3b	42.0 ± 11.0b	0.274 ± 0.029b
Frankia (A)		***	**	***
AMF (B)		*	**	***
A × B		***	**	***

The values are means of 10 replicates ± SEM. Means followed by the same letters within each column are not significantly different according to Duncan's multiple range test at the level of $P < 0.05$. For two-way ANOVA: *significant effect at the level of $P < 0.05$, **significant effect at the level of $P < 0.01$, ***significant effect at the level of $P < 0.001$.

significantly increased by 478% in plants inoculated with *Frankia* spp. alone compared with the uninoculated controls. This significant increase in chlorophyll $a + b$ content was even greater in dual inoculated plants since there was an increase of 531% compared with the uninoculated controls (Table 3). When a dual inoculation was performed, the rhizosphere pH after plant growth was significantly decreased to values closer to pH 7 more than in any of the other treatments (Table 3).

Defoliation experiment

The number of leaves, total leaf area and total leaf dry weight of the plants after experimental defoliation were significantly greater in dual inoculated plants when compared with all the other treatments (Table 4). There were no significant differences in any of the measured parameters between uninoculated controls, plants inoculated with *Frankia* spp. alone and plants inoculated with *G. intraradices* alone.

Frankia and mycorrhizal parameters

The *Frankia* inoculum used induced normal nodule formation in *A. glutinosa* seedlings in highly alkaline conditions. No nodules were observed in uninoculated controls and in plants inoculated with *G. intraradices* alone. In one hand, the number of *Frankia* nodules, dry weight per plant and the dry weight per nodule were significantly increased in the presence of *G. intraradices* (Fig. 1). On the other hand, the density of AMF spores and the ERM vitality were significantly increased in the presence of *Frankia* (Table 5). The ERM length measured by two different methods (IMT and MFT) was significantly greater when a dual inoculation was performed in comparison with single inoculation with *G. intraradices*. There was no significant influence of *Frankia* on the %RLC by AMF. No AMF root colonisation was observed in uninoculated controls and in plants inoculated with *Frankia* spp. alone. The ERM length measured by IMT and MFT showed a significant positive correlation ($r^2 = 0.73$, $P < 0.001$).

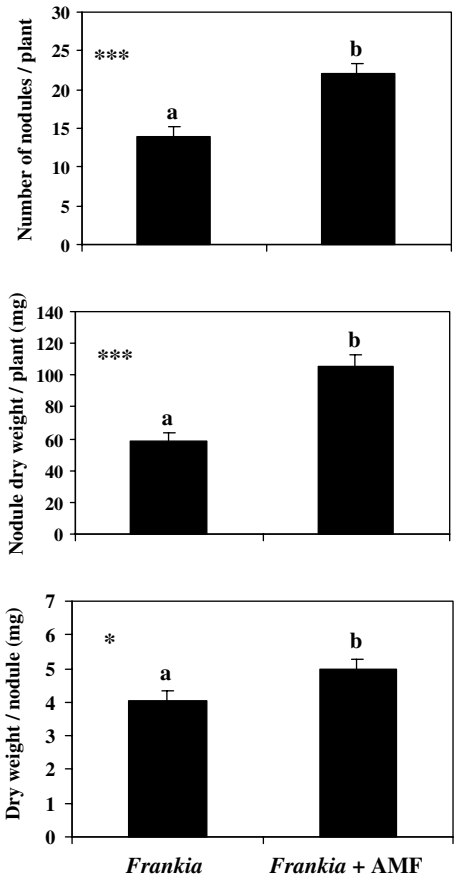

Fig. 1. Effect of *G. intraradices* on the nodulation by *Frankia* spp. of *A. glutinosa* roots grown in a highly alkaline anthropogenic sediment. The values are means of 20 replicates ±SEM. Columns marked with different letters are significantly different according to Student's *t*-test at the level of $P < 0.05$. *Significant effect at the level of $P < 0.05$, ***significant effect at the level of $P < 0.001$.

Table 5
Effect of *Frankia* spp. on the development of the arbuscular mycorrhizal symbiosis between *G. intraradices* and *A. glutinosa* grown in a highly alkaline anthropogenic sediment

Treatment	AMF colonisation (%RLC)	Number of spores (spores g^{-1} dry soil)	ERM length by MFT (cm g^{-1} dry soil)	ERM length by IMT (cm cm^{-2})	NADH diaphorase activity (%)
G. intraradices	86 ± 1	25 ± 1.7	442 ± 71	15.3 ± 1.9	19 ± 2
Frankia spp. + *G. intraradices*	85 ± 1	34 ± 1.6	726 ± 50	27.3 ± 2.7	39 ± 6
Student's *t*-test significances	NS	**	**	**	**
n	20	20	10	10	10

The values are means ± SEM. **Significant effect at the level of $P < 0.01$, NS—non-significant effect, RLC—root length colonised, MFT—membrane filtration technique, IMT—inserted membrane technique.

Discussion

The results from this study show that dual inoculation with *G. intraradices* BEG163 and *Frankia* spp. can greatly increase the growth and improve the nutrition of *A. glutinosa* in a highly alkaline sediment more than single inoculation with either symbiont. Despite the fact that only *G. intraradices* was indigenous to the study site, both microbial symbionts were able to survive and establish a functional tripartite symbiosis with *A. glutinosa*. The ability of *Frankia* spp. to survive and nodulate under extreme pH conditions may be greater than previously reported (Batra, 1991). Individually, *Frankia* had a greater effect on plant growth than the mycorrhizal fungus. This result is in agreement with the findings of other authors in experiments with different *Alnus* species: *Alnus incana* (Chatarpaul et al., 1989) and *Alnus acuminata* (Russo et al., 1993). This may be due to the increased N nutrition observed in alder plants inoculated with *Frankia* spp. when compared with uninoculated controls or plants inoculated with *G. intraradices* alone. The *Frankia* capability for N fixation may play a key role for nodulated alders growing in the extremely alkaline sediment tested. The increased levels of N, P and K in leaves with both endosymbionts present, indicates their effectiveness in improving the nutrition of *A. glutinosa*. The increased N leaf content of dual inoculated *A. glutinosa* compared with plants inoculated with *Frankia* spp. alone may be explained by the increased P provision by the fungus, since large amounts of this element are required for nitrogen fixation (Jha et al., 1993). *G. intraradices* may have also contributed directly to N uptake (Monzón and Azcón, 2001), but this only happened in nodulated alders.

In the defoliation experiment, only dual inoculated trees produced significantly greater number of leaves, total leaf area and total leaf dry weight when compared with the uninoculated controls. This shows that not only the trees forming both mycorrhizas and actinorhizas had improved growth and nutrition, but also they were able to recover faster from an environmental stress (e.g. defoliation by herbivores). The processes of faster leaf regrowth and the production of greater numbers of leaves and leaf areas may be related to greater energy reserves (P) in dual inoculated plants. These plants also produced larger leaves with greater chlorophyll $a + b$ content indicating their increased potential for photosynthesis. It is known that colonisation by AMF alters the internal levels of growth phytohormones of host plants (Ludwig-Muller, 2000) and *Frankia* nodules also contain plant growth hormones (Mansour, 1994). It is therefore possible that in dual inoculated plants the hormonal levels were altered and promoted a faster leaf regrowth.

The rhizosphere of plants hosting both symbionts was also significantly less alkaline than when present alone or in plants without symbionts. The rhizosphere of *G. intraradices* inoculated plants was permeated by ERM and it has been reported that its activity is accompanied by a rhizospheric pH decrease (Bago et al., 1998) as occurred here. The contribution of *Frankia* to the observed rhizosphere acidification may have also been an indirect effect due to the improved growth of the *Frankia* spp. inoculated plants, which in turn may have produced more root exudates that decreased the rhizosphere pH. Given that phosphate is usually as its maximum solubility at near neutral pH (Lindsay, 1979) and that the rhizosphere pH in dual inoculated plants was significantly closer to neutral than in any other treatment, this may explain the highest P acquisition observed in these plants when compared with all other treatments. The dual inoculation resulted in a larger size and greater number of root nodules than inoculation with *Frankia* spp. alone, which is in agreement with previous findings (Fraga-Beddiar and Le Tacon, 1990; Struková et al., 1996; Tian et al., 2002). The concomitant better development of *Frankia* nodules and associated AMF may be explained by the fact that bigger plants were obtained when a dual inoculation was performed. The drain on plant photosynthates by the two symbionts is likely to have been higher and in dual inoculated plants there was probably a larger amount of plant-derived carbohydrates available to the microbial symbionts promoting their development.

Our work is the first to report an increase in the ERM length and NADH diaphorase activity of mycorrhized plants due to the additional inoculation with *Frankia* spp. The significant positive correlation between the ERM length measured by IMT and MFT indicates that the two methods provided comparable data. Further studies on the mechanisms involved in the interactions between *Frankia* and the development of ERM by AMF are needed.

This study shows that *G. intraradices* and *Frankia* spp. can tolerate high pH anthropogenic sediments and assist in alleviating the stress of high pH by promoting nutrient uptake and growth of *A. glutinosa*. The potential of these symbionts for promoting alder growth is great in polluted soils and can be extended for applications in sites with high pH conditions. Dual inoculations must be considered in the future as an additional biotechnological tool for restoration programs of anthropogenic industrial sediments with adverse conditions. The remediation model using *A. glutinosa* together with inoculated bacterial and fungal symbionts is proposed for the phytorestoration of such anthropogenic stressed sediments with highly alkaline pHs.

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